KU 812: A Pluripotent Human Cell Line With Spontaneous Erythroid Terminal Maturation

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A human leukemic cell line KU 812 was recently established and described as a basophilic cell line. In the present study we show that KU 812 and two of its clones are at least bipotent: in addition to a minor component of basophils, the majority of KU 812 cells belongs to the erythroid cell lineage with a significant percentage (about 15%) of mature hemoglobinized erythroblasts. This terminal differentiation is associated with the synchronized synthesis of the main erythroid proteins, including globin chains, spectrin β chain, band 3, and hemoglobin. The predominant hemoglobin is adult, fetal, and Bart’s hemoglobin. Adult hemoglobin represented up to 75% of all hemoglobins in the KU 812 F clone in passages containing a high number of mature erythroblasts. Transcripts of all human globin chains were present with ten times less embryonic chain messenger RNA (mRNA) than α-, β- or γ-chain mRNA. Hemin slightly increased the total hemoglobin production of the cell line, especially γ-globin chain synthesis, but did not modify the percentage of hemoglobinized cells. Phorbol myristate acetate (PMA) had a complex effect, inducing a proportion of KU 812 cells to adhere to the plastic culture flask. The adherent cell fraction expressed a very low level of specific erythroid proteins, but their ultrastructure was consistent with immature erythroid cells. In contrast, approximately 40% of the nonadherent cells were mature erythroid cells. Cell-sorting experiments showed that this paradoxical effect of PMA is mostly due to cell selection, the more mature cells being unable to adhere. In addition, KU 812 F was found to be sensitive to erythropoietin, which slightly increased its plating efficiency range (from 0% to 50%) in semisolid medium and enhanced hemoglobin accumulation twofold. In binding experiments using 125I-erythropoietin, a single class of high-affinity Epo receptors (Kd: 250 ± 120 pM) was detected by binding with a density of 205 receptors per cell. The KU 812 cell line is therefore a unique model for studying cell commitment toward different hematopoietic lineages and erythroid differentiation.

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Hematopoietic tissue is characterized by a continuous process of cell differentiation. A small proportion of cells in normal marrow are capable both of self-renewal and of differentiation along one of the hematopoietic cell lineages. However, studies of human hematopoiesis have been impeded by the difficulty of maintaining sufficient numbers of normal stem cells in culture. Permanent cell lines may be an attractive alternative if they can mimic normal processes, (i.e., sustained self-renewal associated with differentiation toward different lineages and sensitivity to hematopoietic growth factors). However, human cell lines presently available, such as HL 60, K 562, HEL, and LAMA 84, although able to sustain self-renewal, are not capable of terminal differentiation toward several lineages. HL 60 behaves like a cell line committed toward the monocytic and granulocytic lineages. K 562, HEL, and LAMA 84 are erythroleukemic cell lines that might be pluripotent. It is noteworthy that in these cell lines the same cells may coexpress antigens that are usually associated with either the granulomonocytic or the megakaryocytic lineages and erythroid-specific proteins. Terminal differentiation of all these cell lines appears limited even after induction. There is an obvious interest for cell lines much closer to normal hematopoiesis.

The KU 812 cell line was obtained from a patient with chronic myeloid leukemia and was initially described as a basophilic cell line. Basophilic differentiation in KU 812 cells was demonstrated by the presence of metachromatic granules after toluidine blue staining and by the detection of histamine, as well as by the obtaining of basophilic polymorphs in serum-free cultures. In the present study we show that this erythropoietin (Epo)-sensitive cell line is also able to differentiate spontaneously toward the erythroid lineage until the stage of an acidophilic erythroblast. This differentiation is associated with the synthesis of the main erythroid proteins, including adult and fetal hemoglobins.

Materials and Methods

Cell lines and culture conditions. The KU 812 cell line and two of its clones, KU 812F and KU 812E, were grown at 37°C in RPMI 1640 (Boehringer, Mannheim, FRG) containing 10% (vol/vol) fetal calf serum (FCS), bovine) in a fully humidified atmosphere with 5% CO2. Passages were performed every four days, diluting the cells four times. Some liquid cultures were also performed in serum-free conditions (Iscove’s modified Dulbecco’s medium [IMDM] plus iron-saturated transferrin).

Semsolid assays. KU 812 cells were grown in methylcellulose cultures either in the presence of 10% FCS or in serum-replaced conditions by using the previously described techniques, as applied to human cells. Briefly, the medium contained 1.5% deionized serum albumin (Cohn fraction V, Sigma Chemical Co, St Louis), iron-saturated human transferrin (300 μg/mL, Sigma), calcium chloride (28 μg/mL), a mixture of sonicated lipids, 7.5 × 10−4 mol/L α-thioglycerol, 100 mg/mL insulin, and 0.8% 4,000 cp methylcellulose in IMDM. The mixture of lipids was obtained by sonication 7.8 mg cholesterol, 6.14 mg oleic acid, and 7.4 mg dipalmitoyl lecithin (all obtained from Sigma) in 10 mL of IMDM (without sodium bicarbonate) containing 1% serum albumin. Twenty microliters of this mixture was used per milliliter of culture.

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Cultures were performed in pentaplicate or quadruplicate in 1-mL volume in a petri dish (3001, Falcon, Grenoble, France) at a cell concentration of 2.10^6 cells for KU 812 and 5.10^6 for KU 812 F.

**Stimulating factors.** Purified recombinant human GM-CSF and recombinant gibbon interleukin-3 (IL-3) obtained as supernatant of COS-1 cells transfected with the IL-3 gene were obtained from Dr S. Clark (Genetics Institute, Cambridge, MA). Recombinant human Epo was purchased from Amersham (Paris, France). The hematopoietic growth factors were used singly or in combinations.

**Chemicals.** Dimethylsulfoxide (DMSO), phorbol-12-myristate-13-acetate (PMA), hexamethylene bisacetamide (HMBIA), and hemin were all purchased from Sigma Chemicals. They were added to the culture at a concentration of 0.5% to 1% (vol/vol) for DMSO, 8 nmol/L to 160 nmol/L for PMA, 0.5 nmol/L for HMBIA, and 100 μmol/L for hemin.

**Cytotoxic staining.** Cells were observed with May-Grünwald and toluidine blue staining. The percentage of hemoglobin-containing cells was determined by the benzidine reaction as previously described.

**Immunologic markers of differentiation.** A panel of monoclonal antibodies (MoAbs) and polyclonal antibodies recognizing both cell-surface and cytoplasmic markers of differentiation was used. This study was focused on markers related to erythroid differentiation. The MoAbs recognizing the cell surface antigens were anti-EpL, FA6-152, CLB-Ery 3 (anti-blood group H), MR 4-130 (antiglycophorin C [GPC]), and CLB-Ery 1 and R 18 (antiglycophorin A [GPA]). Rabbit polyclonal antibodies against carbonic anhydrase 1 (CA1), spectrin β chain, and band 3 were also used. The expression of hemoglobin (Hb) at cellular level was investigated using an MoAb against the β-globin chain and two polyclonal antibodies directed respectively against the β- and γ-globin chains.

In addition, commercial antibodies against B, T, and “myeloid” differentiation antigens (CD2, CD3, CD10, CD13, CD14, CD15, CD19, CD20, CD33, CD34) were tested as well as antibodies directed against platelets IgG Hb (Tab), GP IIa (C17), and GP Ibα (AN 51). A polyclonal antibody was used to detect myeloperoxidase.

**Indirect immunofluorescence for cell-surface antigens identified by MoAbs** was performed by incubating unfixed cells with an appropriate dilution of the antibody. Cells were subsequently stained with a sheep antimouse IgG + F(ab)2 fragment labeled with fluorescein isothiocyanate (Bioart, Meudon, France) and examined by fluorescence microscopy or flow cytometry. For all the other antibodies, indirect immunofluorescence was performed on cytopsin cell preparations after one minute fixation in methanol.

**Cell sorting.** The KU 812 cell line was labeled with CLB-Ery 1 (anti-GPA) as above under sterile conditions. Cells were sorted into three fractions according to their membrane antigenic density for GPA using a FACS II (Becton Dickinson, Mountain View, CA). Sorted cells were subsequently cultured for two days in the absence or presence of 80 nmol/L PMA and reanalyzed at day 2 of culture for their GPA expression after labeling by CLB-Ery 1 using the FACS II.

**Cell cycle.** Cell cycles were studied by analysis of propidium iodide (Sigma) staining using a flow cytometer as described elsewhere. Briefly, cells were fixed in 50% methanol, rehydrated, incubated in a solution (50 ng/mL) of RNase (Boehringer), and subsequently resuspended in a propidium iodide solution at 4°C for 30 minutes and analyzed using an ATC 3000 (ODAM, Wissembourg, France).

**Ultrastructural cytochemistry and immunogold staining.** Cells in suspension were fixed using 1.25% glutaraldehyde in Gey's buffer for ten minutes, washed, and incubated in a diaminobenzi-

dine medium. Cells were then postfixed using osmium tetroxide, dehydrated, and embedded in epon. Thin sections were examined with a Philips CM 10 electron microscope, first without staining to detect weak peroxidase activity and ferritin molecules and then after lead citrate staining. For immunogold, 10^6 fixed cells were incubated for one hour at 4°C with AN 51 or C 17 ascitic fluid at 1/25 or 1/200 dilution. After three washes in Tris-buffer, cells were incubated for one hour at 4°C with 200 μL of goat antiguinea IgG antiserum coupled to 40 nm gold particles (Janssen Pharmaceutica, Beerse, Belgium) and washed three times. Cells were then treated as above.

Double labeling was performed with C17 and a rabbit polyclonal antibody against glycogenin using goat immunoglobulins against both mouse Ig coupled to 15-nm gold particles and against rabbit IgG coupled to 5-nm gold particles.

**Hemoglobin and globin chain studies.** Analysis of Hb produced by the cell line was analyzed by both cellulose acetate electrophoresis and ion exchange HPLC. Briefly, cells were frozen at –80°C, thawed, and centrifuged at 12,000 g for 20 minutes. The supernatant was loaded on an HPLC column (Aquaapor, CX 300, Brownlee, CA, 1 mm x 250 mm) under the following conditions: flow ratio 0.08 mL/min, equilibrium with developer A (Bistris 48 mmol/L, KCN 3 mmol/L, pH 6.20). Hemoglobins were eluted in a linear gradient from 0% to 100% of developer B, similar to A but adjusted to 0.2 mol/L with NaCl and pH 6.65. The optical density at 418 nmol/L was recorded and integrated. The hemoglobin fractions were characterized by the elution time using adult, fetal, and embryonic hemoglobin as references. The globin chains were analyzed by urea triton polyacrylamide gel electrophoresis (UT-PAGE-γ) as modified by Rouyer-Fessard et al.

**Rna blot hybridization.** Total cellular RNA was isolated according to the method of Chomczynsky and Sacchi. Aliquots (20 μg of RNA per lane) were size fractionated by formaldehyde/agarose gel electrophoresis and were then transferred to nitrocellulose filters. cDNA hybridization probes were labeled to a specific activity of 1.10^9 cpm/μg. Following incubation at 42°C in the presence of 50% formamide, blots were washed four times in twofold concentrated standard saline citrate (SSC: 150 mmol/L NaCl/15 mmol/L sodium citrate)/0.1% NaDodSO4 at room temperature and twice in 0.1-fold concentrated SSC/0.1% NaDodSO4 at 50°C. Autoradiography was performed overnight or for 96 hours using Kodak X-omat AR film (Kodak Pathe, France).

**Epo receptor characterization.** The methods for binding studies have been previously fully described and validated elsewhere. Briefly, highly purified recombinant human Epo was iodinated with a specific activity ranging from 400 to 1,700 Ci/mmol using the iodogen method. Binding was initiated by the addition of 10^6 to 10^7 cells in 100 μL of Eagle's minimum essential medium containing 10% FCS, 0.02% sodium azide, and various concentrations of labeled Epo in the absence or presence of a great excess (at least 100 x) of unlabeled Epo. Cells were then washed three times with ice-cold phosphate-buffered saline (PBS), and the cell-associated radioactivity was measured by γ counting. Specifically bound radioactivity was calculated by subtracting the radioactivity associated with cells incubated with an excess of unlabeled Epo (nonspecific radioactivity) from that of cells incubated with labeled Epo only (total radioactivity). Equilibrium experiments were analyzed according to Scatchard using an unweighted least-square linear regression fitting.

**RESULTS**

**Cell culture and cytologic characteristics.** The KU 812 cell line and its two clones, KU 812 E and KU 812 F, were cultured in suspension and had a doubling time of about 36
hours. A number of small red cells were observed in the flasks, especially in the KU 812 F clone. In cytospin cell preparations colored by May-Grünwald staining, most cells had the morphological characteristics of blast cells; however, some small cells (from 2% to 15%) had the appearance of acidophilic erythroblasts.

For characterization, cells were first stained by the benzidine reaction in the cell suspension; 14%, 35%, and 45% benzidine-positive cells were detected in the KU 812 cell line and in the KU 812 E and KU 812 F clones respectively. KU 812 cells were further investigated by ultrastructural cytochemistry, which showed that a small fraction (1% to 2%) were basophilic promyelocytes (data not shown). These basophils were identifiable by their typical granules, which contained peroxidase activity, that were also present in the endoplasmic reticulum and the Golgi apparatus. However, the great majority of cells belonged to the erythroid lineage. A wide range of maturation steps was observed (Fig 1A).

The most identifiable cells had morphological features of a “CFU-E-like” cell (ie, a cell containing ferritin molecules presents both diffused in the cytoplasm and localized in a special structure, the theta [θ] granule: Fig 1A, lower insert). Peroxidase activity identical to platelet peroxidase (ie, present in the endoplasmic reticulum and the perinuclear space and absent from the Golgi apparatus) was also observed in these cells. Such cells had previously been found in early erythroid leukemia and in marrow cell fractions enriched in CFU-E.34 The most mature erythroid cells of the KU 812 line were hemoglobinized erythroblasts that had lost most of their cytoplasmic organelles (Fig 1A). Thus spontaneous erythroid maturation up to the acidophilic erythroblast is present in this cell line. Similar results were obtained with KU 812 of various sources (Japanese Cancer Resources Bank [Tokyo] and Dr K. Kishi [Niigah, Japan]). The erythroid maturation of KU 812 cells was further characterized by investigation of the erythroid proteins they produced.

Expression of erythroid markers of differentiation by KU 812 cells. Cells were studied by immunofluorescence using several antibodies directed against erythroid markers of differentiation (Table 1). The main erythroid proteins such as CA 1, hemoglobin, spectrin β chain, band 3, GPA, and GPC were detected. The expression of all these erythroid proteins increased with maturation; especially small cells were brightly labeled by all the antibodies, whereas larger cells were either negative or faintly labeled, depending upon the antibody. The anti-Ep I, the anti-blood group H, and the FA6-152 MoAbs recognizing antigens present on normal erythroid progenitors reacted with up to 90% of KU 812 cells.

The expression of globin chains was investigated by immunofluorescence using antibodies directed against the β- and the γ-globin chains. The MoAbs and polyclonal antibodies against the β chain and the polyclonal antibody

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Fig 1. Characterization of the KU 812 cells by ultrastructural techniques and immunofluorescence. (A) Erythroid cells of the KU 812 line with the presence of large blast cells and fully hemoglobinized erythroblasts (original magnification x2,860.) Lower insert: Detail of the cytoplasm of a large blast cell showing a θ granule containing ferritin molecules (original magnification x 74,000.) Upper insert: An aggregate of small cells with the appearance of acidophilic erythroblasts stained by the anti-β-globin chain antibody. Double staining with the anti-β-globin chain (fluorescein) (B) and the anti-γ-globin chain antibodies (rhodamine) (C). Staining with the anti-β-globin chain antibody is intense in the small cells, whereas large and small cells are labeled with the same intensity by the anti-γ-globin chain antibody. A small β-globin chain-positive cell is negative for the γ-globin chain. On these photographs most of the large cells labeled by the anti-γ-globin chain antibody appear unstained by the anti-β-globin chain antibody. In fact, they are weakly stained by this antibody when observed under a fluorescent microscope.
Table 1. Erythroid Markers of Differentiation in the KU 812 Cell Line

<table>
<thead>
<tr>
<th>Erythroid Markers of Differentiation</th>
<th>KU 812 %</th>
<th>KU 812 F %</th>
<th>KU 812 E %</th>
<th>PMA-induced KU 812 Adherent</th>
<th>Nonadherent</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPA</td>
<td>70</td>
<td>70</td>
<td>75</td>
<td>3</td>
<td>60</td>
</tr>
<tr>
<td>GPC</td>
<td>16</td>
<td>45</td>
<td>76</td>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td>Blood group H</td>
<td>98</td>
<td>93</td>
<td>79</td>
<td>93</td>
<td>97</td>
</tr>
<tr>
<td>CD36</td>
<td>95</td>
<td>80</td>
<td>100</td>
<td>75</td>
<td>71</td>
</tr>
<tr>
<td>EP 1 antigen</td>
<td>95</td>
<td>97</td>
<td>90</td>
<td>4</td>
<td>23</td>
</tr>
<tr>
<td>CA 1</td>
<td>96</td>
<td>93</td>
<td>93</td>
<td>90</td>
<td>92</td>
</tr>
<tr>
<td>Benzidine reaction</td>
<td>15</td>
<td>45</td>
<td>38</td>
<td>9</td>
<td>35</td>
</tr>
<tr>
<td>β-globin chain</td>
<td>13</td>
<td>49</td>
<td>42</td>
<td>13</td>
<td>39</td>
</tr>
<tr>
<td>γ-globin chain</td>
<td>17</td>
<td>68</td>
<td>74</td>
<td>8</td>
<td>36</td>
</tr>
<tr>
<td>Spectrin (β chain)</td>
<td>42</td>
<td>62</td>
<td>75</td>
<td>9</td>
<td>48</td>
</tr>
<tr>
<td>Band 3</td>
<td>54</td>
<td>77</td>
<td>80</td>
<td>11</td>
<td>50</td>
</tr>
</tbody>
</table>

KU 812, KU 812 E, and KU 812 F cell lines were grown in the absence or presence of 80 nmol/L PMA. Indirect immunofluorescence was performed on unfixed cells with MoAbs and after methanol fixation with polyclonal antibodies. The MoAbs were anti-Ep1,11 FA6-152,12 CLB-Ery-3,13 and MR 4-13014 directed respectively against the EP 1 antigen, CD 36, blood group H antigen, and a glycosylation-dependent epitope of GPC. Two MoAbs, R 1815 and CLB-Ery-1,13 and a polyclonal antibody against GPA were used. All the other erythroid proteins were detected with monospecific polyclonal antibodies except the β-globin chain, which was also studied with a MoAb.17 Binding of each antibody was revealed with a sheep antirabbit IgG + M Fabβ fragment conjugated to fluorescein or a goat antirabbit IgG Fabβ fragment conjugated to rhodamine for MoAbs and polyclonal antibodies respectively. Cells were examined under a Zeiss fluorescence microscope. Results are expressed as the percentage of positive cells. All experiments were repeated at least four times.

against the γ chain stained a similar proportion of KU 812 cells; a higher percentage of positive cells was always detected in KU 812 F than in KU 812 E or KU 812 cell lines (for β-globin chain: 13%, 42%, 49% of KU 812, KU 812 E, and KU 812 F respectively in the first few passages). Staining of KU 812 cells by anti-β-globin chain antibodies was intense in all small cells (Fig 1, upper insert). Double staining using indirect immunofluorescence was performed between the anti-β-globin chain MoAb and the anti-γ–globin chain polyclonal antibody. The majority (82%) of the cells that expressed hemoglobin were labeled by both antibodies. However, 20% of these cells were only stained by the anti-γ–globin chain antibody; these were large cells with low hemoglobin content (Fig 1C). Two percent of the hemoglobinized cells were only stained by the anti-β–globin chain (Fig 1B, arrow). Some differences in the pattern of labeling were observed between the two antibodies. The intensity of labeling with the anti-β–globin chain was inversely correlated with the size of the cells and presumably directly correlated with the erythroid maturation (Fig 1B). This was not observed with the anti-γ–globin chain antibody, which stained large and small cells with the same intensity (Fig 1C).

Usually after 12 or more passages, the percentage of hemoglobinized cells decreased (Table 2). However, the immunophenotype of the cell line remained erythroid, and spontaneous erythroid maturation was recovered by recolonizing the cell line even after more than 28 passages (data not shown). When KU 812 cells were grown for a long period (6 weeks or more) in serum-free medium (medium only supplemented with iron-saturated transferrin), cells lost most of their erythroid features (GPA and hemoglobin) but did not acquire basophilic features of differentiation.

Analysis of hemoglobins was performed by cellulose acetate electrophoresis and ion-exchange high-performance liquid chromatography (HPLC). Both techniques demon-

Table 2. Determination of the Hemoglobin Content of KU 812 F in Different Culture Conditions

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>Hb A</th>
<th>Hb A + Hb F µg/10^6 cells</th>
<th>Hb F µg/10^6 cells</th>
<th>Hb + cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8th passage</td>
<td>0.75</td>
<td>1.38</td>
<td>0.48</td>
<td>45*</td>
</tr>
<tr>
<td>8th passage + hemin</td>
<td>0.58</td>
<td>1.25</td>
<td>0.91</td>
<td>43*</td>
</tr>
<tr>
<td>15th passage</td>
<td>0.69</td>
<td>0.81</td>
<td>0.38</td>
<td>20*</td>
</tr>
<tr>
<td>15th passage + Epo</td>
<td>0.63</td>
<td>1.5</td>
<td>0.88</td>
<td>48*</td>
</tr>
<tr>
<td>18th passage</td>
<td>0.64</td>
<td>0.56</td>
<td>0.32</td>
<td>35†</td>
</tr>
<tr>
<td>18th passage + hemin</td>
<td>0.58</td>
<td>1.10</td>
<td>0.78</td>
<td>34†</td>
</tr>
<tr>
<td>19th passage</td>
<td>0.69</td>
<td>0.60</td>
<td>0.26</td>
<td>35†</td>
</tr>
<tr>
<td>19th passage + hemin</td>
<td>0.66</td>
<td>1.06</td>
<td>0.56</td>
<td>35†</td>
</tr>
<tr>
<td>22nd passage</td>
<td>0.62</td>
<td>0.39</td>
<td>0.24</td>
<td>10*</td>
</tr>
<tr>
<td>22nd passage + hemin</td>
<td>0.45</td>
<td>0.15</td>
<td>0.18</td>
<td>4*</td>
</tr>
<tr>
<td>28th passage</td>
<td>0.36</td>
<td>0.004</td>
<td>0.008</td>
<td>Undetectable*</td>
</tr>
<tr>
<td>28th passage + Epo</td>
<td>0.37</td>
<td>0.007</td>
<td>0.012</td>
<td>&lt;1%*</td>
</tr>
</tbody>
</table>

Hb content was determined by HPLC after lyse of 16.10^6 cells. Cells were induced by hemin (100 µmol/L) or Epo (2 U/mL) for five days.

*Percentage of Hb A studied by immunofluorescence with an MoAb.
†Percentage benzidine positive cells.
‡Serum-free cultures during five passages.
strated that HbF (49%) and, to a lesser extent, HbA (35%) were the two predominant hemoglobins in KU 812 (Fig 2). Bart's Hb (14%) was also present, as were trace amounts of Portland hemoglobin. In KU 812 F, Hb A was the predominant hemoglobin (up to 75%) in the first few cell passages (Table 2).

Globin chain analysis by urea-triton PAGE showed the presence of \( \alpha, \beta, \) and \( \gamma \) chains (Fig 3). The proportion of globin to nonglobin proteins decreased progressively from KU 812 F through KU 812 E to KU 812. No embryonic globin chains were detected, but this technique is not very sensitive in the absence of hemoglobin purification.

Erythroid protein mRNAs were analyzed using Northern blots (Fig 4 A, B, C). KU 812 cells contained the three size species of GPA mRNAs (1.1, 1.7, and 2.4 kb long); Fig 4C previously described in reticulocytes and the K 562 cell line\(^1\) and also shown in control mRNAs of Fig 4C. The mRNAs coding for the erythroid-specific porphobilinogen deaminase isozyme\(^1\) and for GPC were also present (data not shown). High amounts of mRNA for the \( \alpha, \beta \) (Fig 4A) and \( \gamma \) (Fig 4 B) globin chains were found. The KU 812 cell line contained more mRNA for \( \beta \) globin than its two clones, as previously shown at the protein level. In addition, mRNAs of embryonic globin chains (\( \xi \) and \( \epsilon \) globin chains) were detected but in much smaller amounts (about ten to 20 times less) than the adult or fetal globin chain mRNAs.

**Expression of markers of other cell lineages.** All the antibodies tested against various lymphoid antigens (ie, CD 2, CD 10, CD 19, and CD 20) were negative. "Myeloid" antigens CD 13 and CD 33 were present on some KU 812 cells (30% to 40%), whereas CD 14, CD 15, and myeloperoxidase remained undetectable. CD 34 was detected on less than 5% of the cells. During culture, platelet GP Ib and IIa were detected on from 5% to 30% of KU 812 cells. GP Ib \( \alpha \) was detected on less than 1% of the cells.

At the ultrastructural level, most of the GP Ib and GP IIa positive cells corresponded to immature erythroid cells (\( \theta \) granules and ferritin), but occasional cells were evocative of promegakaryoblasts by their high nucleocytoplasmic ratio and a strong platelet peroxidase activity.

**Effect of chemical inducers of differentiation.** Various chemical inducers were tested to determine whether the KU 812 cell line could be further induced. HMBA (0.5 mmol/L), DMSO (from 0.5% to 1%), and hemin (100 \( \mu \)mol/L) had no clear effect on the number of benzidine-positive cells or on the expression of nonerythroid markers of differentiation when studied by immunofluorescence. The effect of hemin was studied in more detail. Overall, hemin did not increase the number of hemoglobinized cells but in two experiments enhanced hemoglobin accumulation (up to twofold; Table 2). Hb F was slightly enhanced, whereas accumulation of HbA diminished in KU 812 F in the eighth passage (Table 2). The same result was found at the mRNA level (Fig 4A and B). Dot-blot analysis by serial dilutions and using the \( \beta \)-actin probe to control the quantities of mRNA revealed that \( \gamma \)-globin mRNA increased by a factor of two (data not shown). This effect was found in the absence of cell-growth modifications. In KU 812 and KU 812 E, hemin also decreased the amounts of \( \beta \)-globin transcripts (Fig 4A) but only had a slight effect on transcription of the \( \gamma \)-globin gene. In parallel experiments, hemin was able to increase hemoglobin synthesis in K 562 cells more than tenfold. Surprisingly,
Fig 4. Northern blot analysis of the β-globin, γ-globin, GPA, and β-actin mRNA in KU 812 and its clones in different culture conditions. Total cellular RNA was isolated from KU 812, KU 812 E, and KU 812 F at the eighth passage and from control cells (ie, K 562, peripheral blood leukocytes stimulated by phytohemagglutinin, and 12-week-old human fetal liver.) RNA (20 μg) was analyzed as in Materials and Methods and sequentially hybridized with 32P fragment first of the Hb1S clone (human β globin gene) and the mouse β actin gene (A), second of the γ - globin gene (B), and third of the G8 GPA cDNA insert24 (C). Lane 1, K 562; lane 2, peripheral blood leukocytes stimulated by PHA: lane 3, KU 812; lane 4, hemin (100 μmol/L)-treated KU 812; lane 5, KU 812 E; lane 6, hemin (100 μmol/L)-treated KU 812 E; lane 7, KU 812 F; lane 8, hemin (100 μmol/L)-treated KU 812 F; lane 9, human fetal liver: lane 10, adherent fraction of PMA-induced KU 812 F; lane 11, nonadherent fraction of PMA-induced KU 812 F.

hemin also modified the synthesis of GPA. In KU 812 and KU 812 E, it slightly reduced the amounts of GPA and mRNAs, whereas it enhanced its accumulation in KU 812 F (Fig 4C). In this latter clone, the surface expression of GPA was slightly increased (25% to 30%) when the cells were analyzed by flow cytometry after staining with anti-GPA MoAb (data not shown).

PMA (8 nmol/L to 160 nmol/L) apparently modified the differentiation of the KU 812 cell line. In a few hours (one or two hours), a fraction of the cells (10% in KU 812 F v 39% in KU 812) became adherent. Cell-cycle analysis showed that during the two first days, 52% of the adherent cells accumulated in the G2/M phase of the cell cycle (Fig 5B, Fried analysis); at day 5, only 7% of the cells were in the G2/M phase of the cell cycle (Fig 5C). A similar effect of PMA on the cell cycle has been described in the HL 60 cell line.42 The adherent KU 812 cells had low amounts of terminal erythroid differentiation markers (Table 1); GPA, globin chains, spectrin β chain, and band 3 were present in 3% to 13% of the cells. However, these cells continued to express early erythroid markers of differentiation such as blood group H antigen, the FA6-152 antigen, and CA 1. In this adherent cell fraction, the amounts of globin chain (Fig 3) and of GPA and globin chain mRNAs were reduced in comparison with uninduced cells (Fig 4). Dot-blot analysis showed that quantities of the β-globin and GPA mRNAs decreased four times as early as two hours after induction (data not shown). GP IIb and GP IIIa were expressed in the majority of cells (50% to 70%); GP Ib was also highly induced and was present in approximately 30% of the cells. At the ultrastructural level, adherent cells, including those stained by anti-GP IIIa antibody and antiglycocalcin antibody, had morphological features of immature erythroid cells (ie, cytoplasmic ferritin molecules, θ granules, and a platelet peroxidase-like activity; Fig 6). Phagocytosis of latex beads was investigated by electron microscopy and showed that a minority (10%) of adherent cells had phagocytosis capacities as previously reported.10 However, no immuno- logic differentiation markers associated with the monocytic series (ie, CD 14, CD 15, and HLA-DR antigen) could be detected on these cells.

In contrast to the adherent cells, a proportion of the nonadherent cells were mature erythroid cells. Indeed, 30% to 40% were small, with morphological characteristics of acidophilic erythroblasts; after fluorescent labeling, 39% of the KU 812 cells were heavily labeled by the anti-β–globin chain antibody. These hemoglobinized erythroblasts under the electron microscope lacked the platelet peroxidase-like activity and were not labeled by anti-GP IIIa or anti-GP Ib antibodies. Hemoglobin accumulation, studied by HPLC and by globin chains analysis, was to 10 to 15 times greater in nonadherent cells than in adherent cells (Fig 3, lanes 6 and 7). Similar differences were found at the level of the mRNAs by Northern blot analysis in KU 812 F (Fig 4A and B). Dot-blot analysis revealed that nonadherent cells contained eight times more β-globin mRNA than the adherent cell fraction after two hours of induction (data not shown). The results of cell-cycle analysis were similar in this cell fraction and in noninduced cells during the two first days of induction (Fig 5A and D), no transitory accumulation in the G2/M phase of the cell cycle being observed. However, at day 5, 75% of the cells were in the Go/G1 phase of the cell cycle (Fig 5E).

To test whether PMA actually induced KU 812 cells or selected them according to their stage of differentiation, KU 812 cells were sorted after labeling with an anti-GPA MoAb. Three fractions were recovered, corresponding to cells with low, intermediate, and high levels of GPA membrane expression. The presence of the β-globin chain varied in parallel with GPA in these three cell fractions. The fractions were cultured in the presence or absence of 80 nmol/L PMA for two days and reanalyzed for GPA membrane expression. In those with initially low or intermediate GPA expression, PMA decreased the expression of GPA twofold. In contrast, the more mature fraction (15% of the KU 812 cells) remained nonadherent, and PMA had no effect on the expression of GPA and hemoglobin.

Effects of GM-CSF, IL-3, and Epo on KU 812 cells. The effects of GM-CSF, IL-3, and Epo were tested on the cloning efficiency of KU 812 and KU 812 F cells in serum and serum-replaced cultures. Plating efficiency was
8% to 12% for KU 812 and 5% to 8% for KU 812 F. Plating efficiency was slightly higher (10%) in serum cultures than in serum-replaced conditions (data not shown). Colonies were optimum at day 10. In the first cell passages when KU 812 were studied in semisolid medium, some colonies were frankly red and had the aspect of normal CFU-E or BFU-E-derived colonies. GM-CSF and IL-3 alone had no effect on the plating efficiency; in contrast, Epo at a concentration of 2 U/mL increased the plating efficiency in both serum or serum-replaced cultures (Table 3). This effect was moderate (average 15%) but was not reproducible at all passages. Higher concentrations of Epo (up to 16 U/mL) did not further increase plating efficiency. In contrast, Epo increased the number of pink-colored colonies.

We subsequently analyzed the effect of Epo on hemoglobin synthesis in KU 812 and KU 812 F grown in suspension. Epo increased the hemoglobin accumulation about twofold without significant modifications of the pattern of hemoglobin synthesis (Table 2). This increase in hemoglobin production was related to a higher number of cells synthesizing hemoglobin, since the percentage of cells labeled by the MoAb directed against the β-globin chain increased in parallel. A similar result was observed in semisolid medium cultures. The effect of Epo on KU 812 cells suggested that a functional Epo receptor was present on these cells. We subsequently investigated the binding of radiiodinated Epo to these cells.

**Evidence for the presence of Epo receptors on KU 812 cells.** Figure 7 shows that KU 812 cells specifically bound $^{125}$I Epo. Specific binding was proportional to cell number up to at least $7 \times 10^6$ cells per assay (Fig 7A). In association-dissociation kinetic experiments, specific binding increased up to two hours of incubation, then remained roughly constant. After the addition of unlabeled Epo, specific binding decreased (Fig 7B). These results indicate that this binding represents the steady state of a reversible reaction and allow the determination of the equilibrium constant by Scatchard's analysis. The results of this experiment are presented in Fig 7C. The straight line in Scatchard's representation indicates the presence of a single class of noninter-
Fig 6. Double immunogold labeling by an anti-GP Ila MoAb (C17) and an antiglycocalcin (GP Ib α) polyclonal antibody of adherent KU 812 cells treated for four days with TPA. This enlargement shows that the cell membrane exhibits both GP Ila (large particles; large arrow) and glycocalcin (small particles, small arrow), but the labeling remains weak. In the cytoplasm, one can see peroxidase activity in the nuclear envelope and in the dilated endoplasmic reticulum (double arrow); a granule with a pale matrix contains ferritin molecules (original magnification x 46,500).

acting high-affinity binding sites. Data from four independent experiments indicate that KU 812 cells express 205 ± 66 receptors per cell with a dissociation constant of 249 ± 111 pmol/L. In one experiment sodium azide was omitted from the incubation medium without affecting the total number of sites or the equilibrium constant of dissociation. Binding sites with the same characteristics were detected on KU 812 F and KU 812 E cells.

DISCUSSION

Using the uncloned KU 812 cell line and two cloned populations, our results show that this cell line contains cells that are at least bipotent with respect to hematopoietic differentiation. As previously reported, some basophilic cells resembling normal basophilic promyelocytes are present but are a minority. The majority of cells belong to the erythroid lineage, including all stages of differentiation to the acidophilic erythroblast. Cells belonging to other cell lineages than the erythroid and basophilic were not clearly detected; in particular, no unambiguous differentiation markers of the

Table 3. Effects of Hematopoietic Growth Factors on the Plating Efficiency of KU 812 F

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<tr>
<td>O</td>
<td>79 ± 4</td>
<td>68 ± 8</td>
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<tr>
<td>Epo</td>
<td>90 ± 4</td>
<td>85 ± 19</td>
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<tr>
<td>GM-CSF</td>
<td>79 ± 10</td>
<td>70 ± 3</td>
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<td>IL-3</td>
<td>74 ± 4</td>
<td>64 ± 4</td>
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<tr>
<td>Epo + IL-3</td>
<td>86 ± 7</td>
<td>ND</td>
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<td>Epo + IL-3 + GM-CSF</td>
<td>88 ± 2</td>
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Abbreviation: ND, not determined.

KU 812 F cells were plated at a concentration of 5,000 cells per milliliter in serum-replaced conditions; five dishes were scored for each determination. Recombinant human Epo, GM-CSF, and Gibbon IL-3 were used at concentrations of 2 U/mL, 100 pmol/L, and 100 U/mL respectively. Results are expressed as the number of colonies per 1.10⁵ cells. A and B are two independent experiments.

Fig 7. Evidence for the presence of Epo receptors on KU 812 cells. (A) Binding of 125I Epo to KU 812 cells. Total binding (□): nonspecific binding (○); specific binding (■). (B) Association and dissociation kinetics of 125I Epo. 5 × 10⁵ cells were incubated with 200 pmol/L 125I Epo. Dissociation was initiated by adding 50 nmol/L unlabeled Epo after one hour incubation. (C) Binding of 125I Epo as a function of the concentration of erythropoietin added. 2.8 × 10⁶ cells were incubated for two hours at 37°C with increasing concentrations of 125I Epo. Main graph: direct representation of total (□), non specific (○), and specific (■) radioactivity bound to the cells. Insert: Scatchard representation of specific binding.
neutrophilic/erythroid lineages such as myeloperoxidase were expressed. KU 812 cells expressed some "myeloid" antigens, such as CD13 and CD33, which are also present on normal hematopoietic progenitors. In addition, we cannot totally exclude that some megakaryocytic differentiation was also taking place. Indeed, a small fraction of KU 812 cells were stained by anti-GP IIb and GP IIIa MoAbs and even rarer cells were stained by an anti-GP Ib α antibody. The proportion of these cells greatly increased after PMA treatment, but they retained ultrastructural features of immature erythroid cells. Interestingly, the majority of erythroleukemic cell lines express some platelet glycoproteins, especially GP IIb. The reasons for this phenomenon are unclear (eg, aberrant expression, lineage promiscuity between the erythroid and the megakaryocytic lineages, or asynchrony in maturation since GP IIb and GP IIIa might be present on stem cells).

Our data thus demonstrate that KU 812 cells are derived from at least a bipotent basophil/erythroid stem cell capable of sustained self-renewal and spontaneous differentiation. However, it cannot be totally excluded that monocyte/macrophage and megakaryocytic differentiation are also present, especially after PMA induction. Using the following criteria also present on KU 812 cells (ie, the presence of GP IIb, GP IIIa, adherence, and some phagocytic properties), it has been suggested that HEL cells are able to differentiate toward megakaryocytic-like and macrophage-like cells. In normal hematopoiesis, there is no proof of a specific association between the erythroid and megakaryocytic lineages, and recent data suggest that commitment of pluripotent stem cells is stochastic, indicating that there is also no specific association between the erythroid and basophilic lineages. However, in the mouse, some normal marrow cell lines that are maintained by the self-renewal and differentiation of a bipotent stem cell restricted to erythroid and mast cell/basophil lineages have been reported.

Spontaneous terminal erythroid differentiation is one of the main characteristics of the KU 812 cell line. A similar phenomenon has recently been described in a new erythroleukemic cell line JK-1. In contrast, K 562 and HEL cells are constitutively blocked at the proerythroblast level. It is also noteworthy that the constitutive pattern of hemoglobin synthesis by KU 812 cells, characterized by the synthesis of both adult and fetal hemoglobins, differs greatly from that of K 562 and HEL but is identical to that of the induced KMOE-2/05 cell line. Embryonic hemoglobins are only a minor hemoglobin component of KU 812. However, hemoglobin studies may be limited in the future by a progressive loss of terminal erythroid differentiation with cell passages, making it necessary to reclone regularly the cell line.

Hemin had a moderate effect on the erythroid features of KU 812 cells, increasing up to twofold hemoglobin synthesis and favoring γ-globin chain synthesis. Surprisingly, PMA apparently facilitated erythroid differentiation of KU 812. In fact, we showed that this effect was paradoxical and was due to an action that depends upon the maturation level of the target cells. In the case of the more immature cells, PMA diminished their erythroid features by inhibiting the synthesis of erythroid proteins, as previously reported for K 562 and HEL cells, blocked their proliferation, and induced a change from suspension to adherence in some of them. On the other hand, PMA had no marked effect on erythroid cells already committed to terminal differentiation. Thereby these cells remained in the nonadherent fraction.

Finally, KU 812 cells are sensitive to Epo. The effect of Epo on cell proliferation is moderate, since plating efficiency was either modified or only slightly enhanced (10% to 50%), depending upon the number of passages, but is much more marked on the hemoglobinization that is increased by a factor of 2. The two erythroleukemic cell lines K 562 and JK-1 have also been reported to be sensitive to Epo. This prompted us to investigate the Epo receptor on KU 812 cells. A single class of high-affinity binding sites for Epo was detected. Several lines of evidence indicate that these binding sites are the Epo receptors. First, using the same methodology, high-affinity binding sites are not detected in nonerythroid cells; second, the dissociation constant is very close to that reported for other Epo-responsive murine cells and other human erythroleukemic cell lines; third, the association and dissociation kinetics are identical to those reported for Epo receptors in mouse cells and for the two human cell lines K 562 and OCI M1. In contrast, two classes of binding sites have recently been detected on the human cell line JK-1, which responds to Epo in a very similar way to KU 812.

In conclusion, the KU 812 cell line should permit both an understanding of the mechanisms that commit a pluripotent stem cell toward erythroid or basophilic differentiation and the investigation of the different steps of erythropoiesis and hemoglobin expression as a consequence of synchronized terminal erythroid differentiation and sensitivity to Epo.

**ADDENDUM**

During the revision of this report, Okano et al have reported that KU 812 cells synthesize both adult and fetal hemoglobin.

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